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OSMOTIC AND IONIC REGULATION IN THE HORSESHOE CRAB *LIMULUS POLYPHEMUS* (LINNAEUS)

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Limulus polyphemus is one of the four living species of horseshoe crabs, a group with a geological history which can be traced back to the Silurian (*Neolimulus*) and even to the Cambrian (Størmer, 1952). The Mesozoic species originally referred to the genus *Limulus* are closely related and similar to present-day forms and *Mesolimulus walchi* of the Jurassic Solnhofen lagoons co-existed with a fauna which included jelly-fishes, swimming crinoids and ammonites (Abel, 1927). During the intervening 135 million years (Howarth, 1964) the xiphosurans of the lagoons and seas covering parts of Germany, France, England, Sweden and N. Africa have disappeared leaving three living genera, one on the east coast of North America (*Limulus*), and the other two on the south-east and east coasts of Asia (*Tachypleus* and *Carcinoscorpius*) (Størmer, 1952).

The American *Limulus polyphemus* is found in coastal and estuarine areas from Nova Scotia to Yucatan, the adults migrating shorewards in spring and summer to spawn intertidally on sandy beaches (Shuster, 1957, 1960). Probably all stages tolerate a moderate reduction in salinity and known populations of *Limulus* occur in areas where there are considerable ranges of salinity, *e.g.*, Miles River, Maryland (salinity 8–17‰), Ashepoo River and St. Helena Sound, South Carolina (18.4–30.6‰), Hales Cove, Plum Island Sound, Massachusetts (6–32‰) (Shuster, 1966, 1969 personal communication), Delaware Bay, New Jersey (7–30‰) (McManus, 1969). However, in Barnstable Harbor, on the north shore of Cape Cod, Massachusetts where nests of eggs are to be found in early summer at mid-tide level (Shuster, 1950), the poor run off is slight in relation to the sea water entering and leaving the embayment, resulting in an essentially marine aquatic environment for all stages of *Limulus*. During 1947–1948 the salinity was 29–32‰ at both low and high tides, exceptionally 20–31‰ during spring freshet conditions (Ayers, 1959).

In normal sea water there is dispute about whether the animal is isosmotic (Garrey, 1905; Dailey, Fremont-Smith and Carroll, 1931) or hyperosmotic (Cole, 1940; and Homer W. Smith, in Cole, 1940). McManus (1969) states that *Limulus* is approximately isosmotic in water of about 25‰ salinity, and finds that large specimens (prosomal width 15 cm or more) in water of about 7‰ were slightly hyperosmotic after 24 hours. Twelve out of twenty young crabs (prosomal width 7.5 cm or less) survived a salinity of 2.5‰ for 127 hours. Longer term results at Woods Hole by Pearse (1928) have shown that small individuals (<10 cm long) survive for 2–3 weeks in 25% sea water (about 8‰), dying in weaker solutions (4‰) within 2 days.

The present work was started at Woods Hole in the summer of 1966, with the object of investigating the salinity tolerance and powers of osmotic and ionic regulation of the horseshoe crab. It was extended to include determinations of the principal osmotic constituents of muscle at different levels of external salinity. Some data on the latter aspect have been published by Bricteux-Grégoire, Duchâteau-Bosson, Jeuniaux and Florkin (1966).

Material and Methods

Specimens were obtained from the M. B. L. holding tanks at Woods Hole, and kept in the laboratory in large tanks of gently flowing sea water at 21–23° C. During the period 25th July to 1st September, 1966, the chloride content of the sea water varied only slightly 18.01–18.15 g/liter, with a mean value of 18.07. Corresponding chlorinity values were 17.62–17.76 g/kg, which are equivalent to a salinity of 31.83–32.09 g/kg, the mean being 31.94. For experiments on the effect of salinity changes, specimens were kept individually in well-aerated sea water in plastic containers, dilutions of the water being made with tap-water (a soft water containing about 11 mg chloride per liter), and higher concentrations being obtained with artificial sea water double the normal Woods Hole concentration.

Animals used were chiefly those of carapace (prosoma) width 9.0–9.7 cm and 83–118 g, also several of about 7 cm width, 32–36 g. Large male adults of 17.9–19 cm width, weighing about 620–700 g, were taken for the comparative analyses of blood and muscle. The appearance of the genital apertures was used to sex the animals (Lochhead, 1950). They were not fed during the experiments or during the period, some days to a few weeks, when they were kept in the holding tanks after being trawled.

Blood was withdrawn by syringe or by pipette after a small incision had been made in the ligament joining prosoma and opisthosoma, and then the serum was separated by centrifugation after aggregation of the cells. Removal of the dorsal exoskeleton in large specimens required the use of an old-fashioned can opener (Lochhead, 1950), before the muscles could be obtained. Those used for analysis were the intertergal muscle and the flexors and extensors of the opisthosoma and telson. After they had been blotted lightly with filter paper, separate samples were taken for the determination of water and inorganic cations, and for making trichloracetic acid extracts, tungstic acid extracts and zinc hydroxide extracts.

Methods for sodium, potassium, calcium, chloride and sulphate were those described previously (Robertson, 1949, 1960a), for magnesium that of Heagy (1948). Procedures used for α-amino nitrogen, proline, trimethylamine oxide, lactate, phosphate, and copper are listed in Robertson (1961). Microdiffusion methods of Conway (1962) were used for ammonium ions of muscle and for the final determination of total non-protein nitrogen after micro-Kjeldahl digestion of samples of the tungstic acid filtrates of muscle. Arginine was estimated in trichloracetic acid filtrates by the methods of Janus (1956) and of Ennor (1957), with good agreement between the two.

Extracellular volume of specimens and of the muscle was determined by injecting into the haemocoele a measured volume of a 10% solution of inulin (Kerfut) in sea water (1 ml for specimens of 100–118 g, 5 ml for a specimen of 634 g). After periods varying from 5 to 10.3 hours in different animals, samples of blood and muscle were removed, zinc hydroxide filtrates prepared, and their content of inulin measured by the procedure of Roe, Epstein and Goldstein (1949). Chloride was estimated in the same filtrates, so that both chloride "spaces" and presumed extracellular (inulin) spaces could be compared.

Water contents were obtained by measuring the differences between fresh and dry weights; plasma samples were dried for 4 hours and muscle for 24 hours at 100–101° C. Animals were weighed to the nearest 0.1 g when the effect of changed external salinities was being investigated.

Measurements of osmotic concentration of blood serum were made thermoelectrically with the Krogh-Baldes thermocouple (Krogh, 1939). Standard deviation of 13 estimations on sodium chloride solutions of approximately the same vapor pressure as sea water was 0.56%.

From the vapor pressure and osmotic equivalence measurements of Robinson (1954) and the osmotic coefficients of sodium chloride from Robinson and Stokes (1959), sea waters of concentrations Cl 10‰ ($\equiv$ 0.2821 M-NaCl), Cl 18‰ ($\equiv$ 0.5263 M-NaCl) and Cl 22‰ ($\equiv$ 0.6503) have the following concentrations in milliosmoles: 528, 970 and 1202. From the freezing-point data of Scatchard and Prentiss (1933) and assuming solutions of sodium chloride and sea water of the same vapor pressure at 25° C have the same freezing points, then the three sea waters would freeze at −0.974° C, −1.780° C and −2.198° C and have the following concentrations (Dick, 1959, Δ 1.858° C $\equiv$ 1000 milliosmoles): 524, 958 and 1184 milliosmoles. Measurements of the osmotic concentration of blood serum and sea water, obtained first as concentrations in relation to the standard drops (3.000 g and 1.000 g NaCl in 100 g water, the one closest to the anticipated concentration being used), were converted to milliosmoles by use of the appropriate osmotic coefficients. Some values for sea water were obtained from the first set of equivalencies above.

Osmotic regulation

(1) *Osmotic concentration and chloride values of blood-serum in relation to external medium.* In Table I are given the results of vapor pressure and chloride analyses of *Limulus* serum from specimens kept in dilutions and concentrations of sea water from 5–200% for periods of 2–15 days, as well as from normal sea water. The results show that in 66% sea water and below *Limulus* osmoregulates, maintaining higher osmotic and chloride concentrations in the blood than in the sea water. Only small *Limulus* could stand the lowest dilution of 5% sea water, those of 32–36 g and about 7 cm carapace width, and then only if acclimatized at intermediate salinities. At this dilution, osmotic and chloride concentrations of the serum were 4–6 times those of the sea water.

In normal sea water *Limulus* is isosmotic, and the values show no significant difference in relation to sex. Taking the ten specimens (♂♂ and ♀♀) together, the mean milliosmolar values of the serum are 955 (S.D. 4.1) compared with 951 for sea water, the range being 949–960. Animals placed in 150% and 200% sea water remained lively and apparently perfectly healthy; they became isosmotic within 1.5% with the new medium.

A linear relationship holds if chloride values are plotted osmotic concentration. Multiplying the chloride concentration by 56.3 for measurements per milliliter

TABLE 1

Osmotic concentration and chloride values of blood-serum in relation to the external medium

Approximate strength of sea water (per cent)	Osmotic concentration			Chloride			Water	Number and sex	Duration in sea water (hours)
	Milliosmoles		Serum as per cent sea water	mg/ml.		mg/g water	mg/ml		
	Sea water	Serum		Sea water	Serum	Serum	Serum		
200	2084	2058	98.8	38.34	36.64	38.85	945	1 ♂	48
200	2006	1976	98.5	37.16	35.06	37.74	929	1 ♀	99
200	1791	1770	98.8	32.80	30.55	32.40	943	1 ♀	71
150	1382	1372	99.3	25.69	24.55	25.55	961	1 ♀	75
150	1376	1392	101.2	25.58	24.37	25.80	945	1 ♀	74
100	950 ± 0.01	954 ± 2.10	100.4 ± 0.22	18.03 ± 0.01	17.48 ± 0.13	18.45 ± 0.12	948 ± 6.8	6 ♂	—
100	951	957 ± 1.19	100.6 ± 0.12	18.04	17.52 ± 0.25	18.44 ± 0.23	950 ± 4.9	4 ♀	—
66	[630]	699	111.0	12.04	12.77	13.38	954	1 ♂	50
66	[630]	718	114.0	12.04	12.82	13.61	942	1 ♀	50
50	[483]	569 ± 41.3	117.9 ± 8.5	9.27	10.20 ± 0.76	10.91 ± 0.83	936 ± 17.0	3 ♂	50; 48 in 80% and 66%
50	[482]	638 ± 21.5	132.3 ± 4.5	9.25	11.43 ± 0.44	12.01 ± 0.45	952 ± 1.0	2 ♀	
33	[323]	476 ± 34.7	147.4 ± 10.7	6.20	8.42 ± 0.52	8.94 ± 0.58	943 ± 11.7	4 ♂	48; 50 in 50%
25	231	372 ± 44.1	161.1 ± 19.1	4.45	6.58 ± 0.90	7.02 ± 0.93	936 ± 6.4	3 ♂	48–50; 24 in 50%
20	187	419 ± 16.0	224.1 ± 8.5	3.61	7.19 ± 0.04	7.68 ± 0.20	937 ± 28.5	2 ♂	48; 50 in 50% and 33%
15	[141]	176 ± 5.0	125 ± 3.5	2.72	2.98 ± 0.12	3.26 ± 0.07	915 ± 15.0	2 ♀	113–144; 46 in 20%
10	[95]	293 ± 6.0	308 ± 6.3	1.84	5.02 ± 0.27	5.26 ± 0.16	953 ± 21.0	2 ♂	50–79; 16–50 in 50%, 33% and 20%
5	[48]	287 ± 34.5	598 ± 71.7	0.92	4.91 ± 0.91	5.07 ± 0.86	961 ± 19.4	3 ♂	40–111; 47–244 in 50%, 33%, 20% and 10%
5	[47]	229	487	0.91	4.23	4.25	996	1 ♀	

[ ] = values calculated from chloride titrations.
± = standard error.

Three of specimens in 50%, two in 33% and all those in lower salinities (except one in 15%) had periods of acclimatization at intermediate salinities, given in last column, after the semi-colon; the first figure is number of hours in the final dilution.

and by 53.2 for those per gram water give calculated values for osmotic concentration differing only slightly from those found experimentally. In both cases the average deviation from the figures in the table is under 2%, and using the 53.2 factor only two of the eighteen calculated concentrations exceed 2.9%. But in calculations of osmotic concentration from serum chloride in the ten specimens in full-strength (100%) sea water, factors of 54.6 and 51.8 are more appropriate, the average deviation from measured values being nil in both cases, with ranges of 0.2–4.1% and 0.1–2.4%, depending on whether volume or water content figures are used.

Figure 1 shows the relationship in osmotic concentration between serum and sea water, after plotting the results for each specimen (30 points, the closely

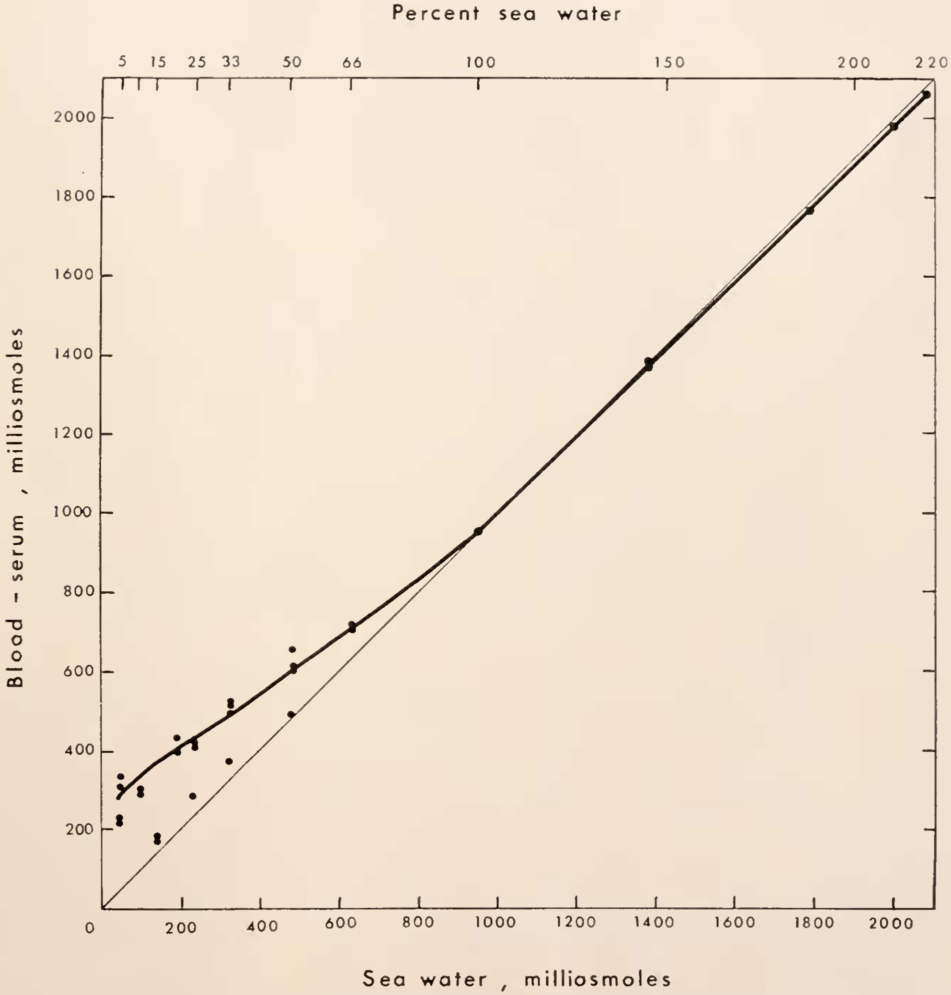

FIGURE 1. The osmotic concentration of *Limulus* blood in relation to that of the external medium.

agreeing values for the ten in 100% sea water being plotted as one point). Five points are near the isosmotic line, all animals of 109–114 g. Four of these animals had been weighed and two particularly, those in 15% sea water, showed increases to 122% and 116% of their initial weight after 6–8 days (the first having been transferred directly to 15% from 100%, the second having a preliminary 2-day period in 20%). Presumably these animals had been overstressed. Of all those in diluted sea water recorded in Figure 1, they were the only ones which did not seem perfectly healthy. When examined out of water the normal resistance to straightening of the flexed opisthosoma had somewhat decreased by the end of the experimental period. Further data on weight changes in general are given in the next section.

Taken as a whole the data in Figure 1 and Table I show that *Limulus* is a hardy animal, able to survive extensive dilutions and concentrations of sea water by maintaining (1) an osmotic steady state at concentrations of about 90–200% sea water, and (2) hyperosmotic regulation at salinities between 5 and 90%. Under experimental conditions the remarkable tolerance of the animal for at least periods of 2–5 days (15 days in the case of the smaller specimens in 5% sea water) is shown by the fact that at the lowest salinity tolerated and at the highest salinity investigated there is a seven-fold difference in blood values of total concentration and chloride.

(2) *Changes in weight and in water content of* Limulus *in different salinities.* Two *Limulus* in 100% sea water were weighed at intervals for 11–15 days. The first of 104.3 g was weighed a further five times, the mean weight being 104.9 g, with a range of 104.3–105.6 g (100–101.2%). The second of 92.4 g was weighed an additional nine times, the mean weight being 91.6 g, range 91.0–92.4 g (98.5–100%).

In Figure 2 are shown percentage changes in weight of specimens transferred directly to dilute and concentrated sea waters over a 10–200% range. Down to a concentration of 33% sea water, weight increases are small, some 2–4%, rather greater in 20% sea water, 5–8%, but quite large, 13–22%, in 15% and 10% sea water. For these medium-sized horseshoe crabs (92–118 g) salinities of 10–15% are critical levels for survival beyond a few days in such direct transfer experiments.

In concentrated sea waters weight was lost, and then regained or partially regained. The two animals in 150% sea water lost 3% of their weight in 6 hr, but regained it in 75 hr, while the three in 200% lost nearly 6% in the same time and none had fully regained original weight when taken for analysis of the blood (the two kept for the longest periods, 71 and 99 hr, attaining 98.3% and 97.6%).

The reversibility of weight increases is shown in Figure 3, where after 84 hr in dilute sea waters of 20% and 15% the weight had increased to 123.5% of the initial value. On return to full-strength sea water, a fairly rapid decrease in weight resulted, apparent in an hour, with a fall to 108.5% in 8 hr and to 100.9% in 22 hr. Thereafter a slight rise occurred to 103.9% 5 days after the return to 100% sea water.

There is a marked difference in total water content of intermolt animals in 100% sea water and in 5%, some 10% difference, which is perhaps slightly exaggerated by a presumed relatively lighter exoskeleton in the younger animals

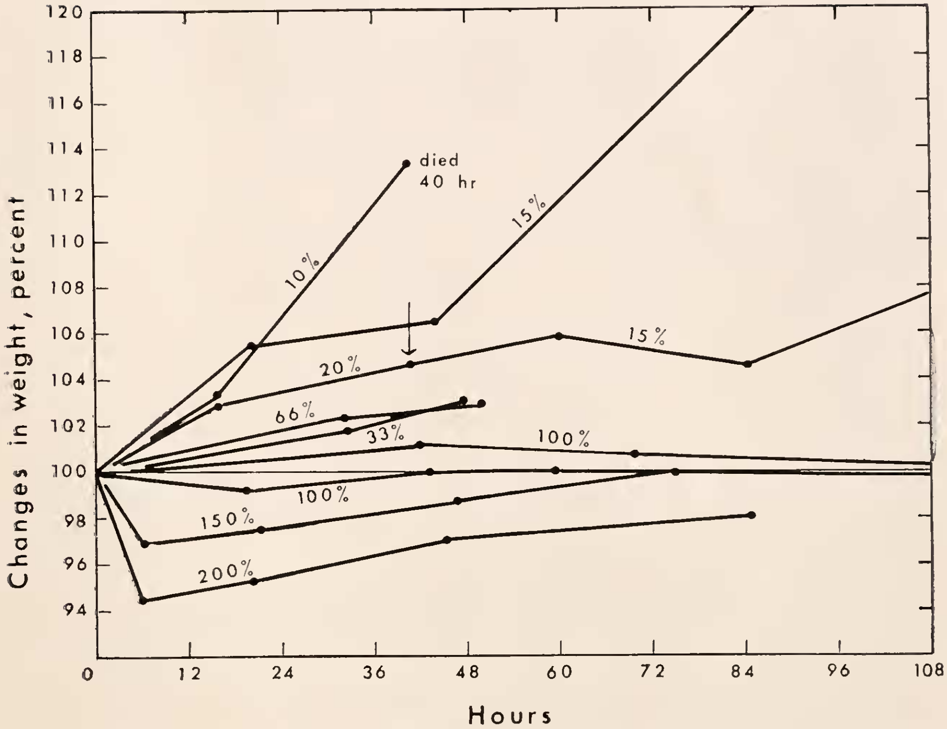

FIGURE 2. Changes in weight of *Limulus* of 92–118 g after direct transfer to different concentrations of sea water. Points are for individual specimens, except in 200% (mean of 3), 150%, 33% and 66% (mean of 2).

(Table II). There is also clear evidence for an increased hydration of the muscles at reduced salinity, animals in sea water of 33% and below having values in the eighties, compared with 79.1–79.7% in 50% and 66% sea water. A reduction in hydration from the normal range of 73.8–77.1% is found in specimens acclimatized

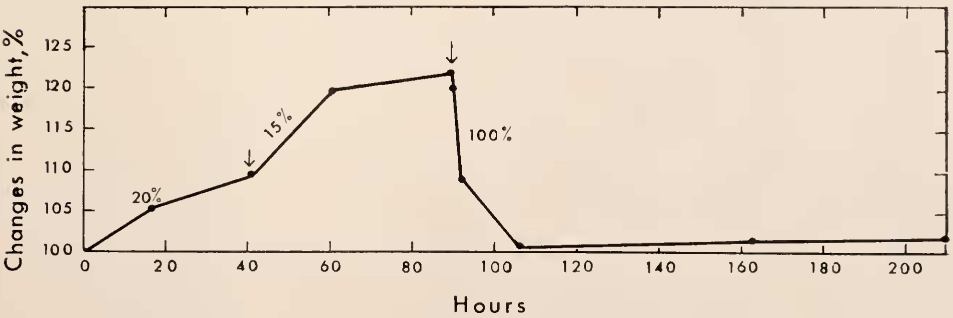

FIGURE 3. Changes in weight of a specimen of 103.8 g in various concentrations of sea water.

TABLE II

Water content of whole animals and of muscle in relation to salinity of external medium (5–200% sea water)

Medium %	Percentage water				Mean serum osmolality (milliosmoles)
	Whole animal	S.D.	Muscle	S.D.	
200	—	—	67.2 (3)	1.86	1931
150	—	—	73.2 (2)	1.71	1382
100	76.8 (3)	1.03	75.2 (4)	1.37	953
66	—	—	79.5 (2)	0.28	709
50	—	—	79.2 (3)	0.41	587
33	—	—	83.0 (2)	0.28	476
25	—	—	82.2 (4)	2.04	372
20	—	—	80.7 (1)	—	435
15	—	—	84.9 (2)	1.20	176
10	—	—	82.5 (2)	1.41	295
5	86.9 (2)	2.55	—	—	272

Weight of specimens mostly within range 102–139 g, but two in 5% were 21.1 and 32.3 g. Serum osmolality and muscle water contents are from the same specimens.

to 150% and 200% sea water, the ranges being 72.1–73.2% and 65.9–69.3%, respectively. The muscles are bathed in interstitial fluid and plasma, not sea water, and from the last column in Table II it is seen that serum osmolality and muscle hydration are inversely correlated (correlation coefficient—0.99). At the extremes of salinity, 100 g solids (chiefly protein and salts) are associated with 205 g water (muscle of 67.2% water) and 471 g (muscle of 82.5% water). In the medium of 15% where the two specimens increased to 116% and 122% of their initial weight, 100 g muscle solids is associated on the average with 562 g water.

TABLE III

Extracellular volume and extracellular water as measured by inulin space

Sex	Prosomal width (cm)	Weight (g)	Extracellular volume		Serum water (mg/ml)	Extracellular water (g/100 g)
			Actual (ml)	Per cent body weight		
♂	18.5	633.8	317.3	50.0	952	47.6
♂	9.5	106.7	52.1	48.8	948	46.3
♂	9.6	104.9	45.8	43.7	940	41.1
♂	9.4	100.3	64.3	64.1	947	60.7
♀	9.7	117.5	67.8	57.8	961	55.5
♀	9.5	101.0	53.8	53.3	950	50.6
♀	9.0	99.6	43.4	43.6	952	41.5
				Mean S.D.	Mean S.D.	Mean S.D.
				51.6 7.47	950 6.35	49.0 7.18

Time allowed for distribution of inulin after injection 5–10.3 hours. The difference in extracellular water between means for the males (48.9 ± S.E. 4.17) and females (49.2 ± 4.10) is not significant according to Student's *t*-test ($P = 0.7$). All specimens were from 100% sea water.

Whatever may be the mechanism for maintaining a more or less constant water content in muscle, it is clear that the general level of hydration is largely dependent on the osmotic concentration of the bathing fluid.

(3) *Extracellular water.* On the assumption that the inulin molecule does not penetrate cells, the data in Table III give an indication of the extracellular volume of specimens of 100–634 g, total extracellular water being then obtained by correcting for the solids in blood-serum. The mean value of 51.6 ml per 100 g for extracellular volume is high compared with a decapod crab such as *Carcinus maenas* in which a mean value of 32.6 ml has been found, using sucrose and inulin (Robertson, 1960b). Two features would partially explain this apparent marked difference: *Carcinus* has a relatively heavy calcified exoskeleton contributing to its weight, and also a lower water content, 65.4%, in the intermolt stage.

It is assumed that the extracellular water, 49.0 g average for a 100 g *Limulus,* includes water of the plasma, interstitial fluid round cells, and perhaps any fluid in the gut and in the coxal glands. On this assumption some 76.8–49 g, or 28 g, is the value for intracellular water and the water of the cuticle.

(4) *Permeability to water and salts.* All the foregoing data point to permeability to water of the body surface, probably mainly the lightly chitinized gills, water passing in when the animal is placed in a hypo-osmotic medium and out when placed in a hyperosmotic medium. But some slight drinking of the media has not been excluded physically by gut ligature. If it occurs it seems to be negligible as no large amount of fluid was seen in the gut during dissections.

Permeability to salts is not so self evident. However, from measurements of chloride in diluted sea water, particularly 5%, before and after specimens had been kept in it, it is clear that some chloride ions pass into the water before a steady state is attained. Permeability to salts can also be inferred from the changes in weight and measurements of osmotic concentration in animals in both stronger and dilute solutions. For example, if the 3 animals put into 200% sea water attained a new osmotic concentration, on average 1935 milliosmoles from an initial value of 955 (Table I), by water abstraction alone, the fall in weight would need to have been about 38% instead of the 6% values found (assuming a 77% water content initially—Table II). Salts must therefore have been absorbed to account for the rise in concentration of the blood to its new equilibrium value with the sea water.

Similarly, salts must have been lost to account for the decreased values of osmotic concentration in all the animals in diluted sea water. Taking the case of the 2 specimens in 33% sea water, a fall in blood concentration from 955 to 476 milliosmoles would require an osmotic intake of 38 g water per 100 g (the original weights were 114 and 117 g). Yet the average weight increase was 3%. Of all the specimens studied, one transferred directly to 15% sea water showed the greatest increase in weight, 94.6 to 115.0 g, 21.6% after 113 hr, and had an osmotic concentration of 181 milliosmoles, the second lowest recorded for any of the experimental animals. The theoretical value for a 100 g horseshoe crab of pure water which would have to be absorbed osmotically to reduce the concentration of blood and tissue water to 181 milliosmoles is (4.28 × 77) ml. It is quite obvious that the reduction cannot be due to osmotic uptake of water but must be effected chiefly by the outward diffusion of salts.

Of 29 *Limulus* tested in diluted sea water (66–5%), there were 7 deaths, all of medium-sized specimens (98–105 g) except one (about 35 g). Four of these, including the small one, died in 36–46 hr after direct transfer to 5% sea water, even though three were active and healthy for the first 25 hr. "Healthy" in this context means showing the normal swimming and aeration movements of the walking legs and opisthosomal flaps, well maintained tone of the flexor muscles, and, in the small one, ability to right itself in a few minutes. The final chloride content of 3 specimens in 5% sea water in different physiological condition after 40 hr, expressed as % of sea water chloride, was 636% (good condition), 331% (poor condition) and 222% (dead). Obvious increases in permeability to chloride have occurred in the last 2 individuals.

The other 3 deaths occurred on direct transfer from sea water to 10% (a 104.9 g animal which increased in weight 13.3% in 40 hr), on transfer to 10% after a preliminary 16 hr in 50% (100.8 g, 18.8% weight increase in 64 hr), and on direct transfer to 15% (98.0 g, 15.4% weight increase in 96 hr). In all 3 cases there was a gradual increase in water uptake until death, not a sudden increase at later stages.

Hardiness of a specimen of 97.2 g (♀) kept in running tap-water was shown by the fact that it increased by only 3.9% in 24 hr. This was partly due to the death and loss of its several epizoic triclad planarians (*Bdelloura*) and the loss of feces, as it had increased by 6.9% at the end of 4 hr. After 25 hr, the limbs still showed movements and the resistance to straightening of the flexed animal outside water had only slightly decreased from normal. Placed on its back in the tank it righted itself in 15 minutes. A small sample of blood showed a chloride value of 5.0 mg/ml, about that of animals living in 5–10% sea water. It was put back into 100% sea water and the subjective observation made that the resistance of the folded opisthosoma was back to normal in 25 minutes. Five hours later the weight had fallen to 99.5% of the original weight and the chloride had risen to 7.98 mg/ml. Inadvertently, the animal was left overnight on the bench after weighing and the final chloride equilibrium could not be followed.

Inorganic ions

(1) *Composition of serum and of flexor and extensor muscles of the prosoma-opisthosoma in animals from normal sea water.* Three comparisons of serum, muscle and the sea water in regard to inorganic ions, lactate and phosphate show fairly good agreement (Table IV). The sum of investigated ions in muscle comes to about half of those in serum and sea water. Apart from higher potassium and lower magnesium and sulphate concentrations in the serum compared with sea water, only small differences in the relative values of sodium and chloride are found, chloride being near the equilibrium value, sodium on average 1% higher, while calcium is higher in two cases, lower in the third.

In muscle half the osmotic concentration must be made up by organic compounds other than those of phosphorus. The increased potassium of muscle, about 9 times the serum value, is offset by lower concentrations of sodium and chloride (about a third to a quarter of serum values), magnesium (about a half), and calcium and sulphate (a half to a third). The average total acid-soluble phosphorus value of 73.3 mg atoms will presumably contain major fractions of arginine phosphate, ATP and hexose phosphates, compounds not separately estimated, acting as anions in the muscle cells. Lactate concentrations were especially high in specimen (2), although no excessive muscular activity had been noted, and the serum value was less than that of the third specimen.

Regarding cation-anion balance in the serum the mean value for cations is 569 milliequivalents, as against 543 for anions. The difference is presumably made up chiefly by protein anions and bicarbonate. For muscle the calculation is difficult, involving several undetermined factors such as the internal pH of the cells which influences the valency of the phosphate compounds, the proportions of the various phosphate compounds, how far the soluble proteins of the cells are acting as anions, and the possible presence of free arginine acting as a cation. Then there is the question of possible ion-binding to cell organelles. Simply assuming a valency of 1 for the total P atoms gives 259 milliequivalents anions as against 274 cations. A higher average valency would be more correct. In the case of the decapod crustacean *Nephrops*, average values calculated from knowledge of its phosphate compounds are 1.23–1.44 depending on the actual cell pH within the range of 6.0–7.0 (Robertson, 1961). Use of an average valency near the lower figure would give apparent cation-anion balance in *Limulus* muscle.

TABLE IV

Ionic composition of Limulus *blood-serum and whole muscle*

	mg-ions/kg water									Water (g/l or g/kg)]
	Na	K	Ca	Mg	Cl	SO_4	Lactate	Total P	Total mg-ions	
♂ (1) Serum	439	10.9	8.8	46.3	507	14.8	0.16	0.15	1027	977
Muscle	147.4	89.0	4.25	22.4	190	—	9.8	58.1	521	771
♂ (2) Serum	441	13.3	10.6	47.5	516	11.6	0.18	0.74	1041	926
Muscle	91.8	106.1	3.20	17.8	142	3.9	35.1	89.6	490	749
♂ (3) Serum	454	11.3	10.2	44.3	519	16.0	0.59	0.40	1056	951
Muscle	139.4	103.4	3.83	21.4	146	—	12.4	72.3	497	770
Means										
Serum	445	11.8	9.9	46.0	514	14.1	0.31	0.43	1041	951
S.D.	8.2	1.3	0.30	1.6	6.2	2.3	0.24	0.30	14.5	25.5
Muscle	126.2	99.5	3.76	20.5	159	3.9	19.1	73.3	503	763
S.D.	30.1	9.2	0.53	2.4	26.6	—	14.1	15.8	16.3	12.4
Sea water	440	9.4	9.7	50.2	514	26.5	—	—	1050	990

(2) *Apparent extracellular spaces in muscle.* Chloride and inulin spaces were obtained by expressing the concentrations of these substances in muscle as percentages of those in serum, both on a water-content basis. The chloride space is a theoretical maximum for total weight or volume of muscle water which is extracellular, on the assumptions that serum has the same chloride content per unit of water as plasma and any interstitial fluid, and that chloride is absent from muscle cells. Any intracellular chloride would result in the chloride space as defined being larger than the extracellular space.

Results in Table V show that the inulin injected into the plasma distributes itself into 22.8% of the muscle water on the average, in 5–10 hours. This mean value is 75% of the chloride space, suggesting that there is some chloride inside the cells. Reasons for accepting the inulin space as a measure of extracellular space in muscle are the large size of the inulin molecule (MW *ca.* 5000), restricting its entry into cells, and its apparent suitability for measurement of extracellular space in such other invertebrates as decapod crustaceans and molluscs (Robertson, 1961; 1965).

The presence of haemocyanin in *Limulus* blood suggests that measurements of copper in muscle and serum will give a maximum value for the amount of blood plasma present in the muscle, but the possibility of other copper compounds in muscle, which would make this maximum an overestimate, must be accepted. Two estimates of copper spaces, expressed as percentage of total muscle water, were 3.5% and 15.4%, not in good agreement. The minimum value for interstitial fluid may be taken as about 13% (22.8–9.5%) of total extracellular water.

TABLE V

Apparent extracellular spaces in Limulus *muscle (concentrations in muscle as percentages of those in serum, on water content basis)*

Specimen no.	Extracellular spaces as per cent total muscle water		Muscle chloride (mg-ions/kg water)	Time allowed for distribution (hours)
	Chloride	Inulin		
1 ♂	27.8	24.9	145.9	5
2 ♂	28.3	19.4	147.5	7.5
3 ♂	28.9	19.4	152.6	8
4 ♂	31.7	25.5	167.3	10.3
5 ♀	30.2	22.3	153.5	6
6 ♀	36.2	25.6	192.1	6
7 ♀	28.8	22.2	152.9	10.3
Mean	30.3	22.8	158.8	
S.E.	1.10	1.01	6.13	

(3) *Intracellular ionic composition of muscle.* Calculations of intracellular concentrations are obtained by subtracting from the concentrations per kilogram muscle water the values for extracellular fluid. Of the two calculations in Table VI, the first one was made by subtracting the values for 249 g extracellular fluid (here taken as serum) from the concentrations per kilogram muscle water of the third specimen in Table IV, in which both extracellular fluid and chemical analyses had been obtained on the same specimen. The remaining concentrations in 751 g muscle water were then expressed as cell values per kilogram water. For the second calculation the means of the other two animals in Table IV were used, in conjunction with the mean extracellular space, 22.8%, of Table V.

The two sets of values for ions in the muscle cells do not differ greatly, those for sodium and chloride the most. Because of the subtraction of sodium- and chloride-rich extracellular fluid, the concentrations of these ions fall to about a quarter of those in whole muscle. Magnesium still remains at a higher intracellular concentration than calcium, although both are reduced. At the same time potassium and phosphate increase by about an eighth. The intracellular potassium concentration now exceeds the sodium, and is approached in concentration only by phosphate; both these features are found in the few invertebrate muscles analyzed in the same way. The final result is a reduced total concentration of ions, about 32% of the serum concentration, compared with 48% in whole muscle.

Comparison of cell/serum ratios in Table VI shows that potassium, lactate and phosphate are accumulated in the cells, the latter especially, as it is the principal anion balancing most of the potassium. Chloride and the three other cations are reduced, magnesium the least.

The mean chloride ratio of 0.084 is not very different from that which would exist if there is a Donnan equilibrium between the concentrations of potassium and chloride inside and outside the cells: $K_i/K_o = 11.0$, $Cl_o/Cl_i = 11.9$. But the mean chloride value inside the cells is derived from two values each of which gives ratios considerably above and below this, so that a simple Donnan relationship is improbable.

TABLE VI

Intracellular composition of muscle compared with blood-serum

	mg-ions/kg water							
	Na	K	Ca	Mg	Cl	Lactate	Total P	Total
Muscle cells								
(1)	35.2	134	1.72	13.8	22.4	16.3	96.2	320
(2)	22.4	123	1.97	12.2	63.9	29.0	95.5	348
Mean	28.8	129	1.85	13.0	43.2	22.7	95.7	334
Serum								
(1)	454	11.3	10.2	44.3	519	0.59	0.40	1040
(2)	440	12.1	9.7	46.9	512	0.17	0.45	1021
Mean	447	11.7	10.0	45.6	516	0.38	0.43	1031
Ratio: $\frac{\text{cell}}{\text{serum}}$	0.064	11.0	0.19	0.29	0.084	59.7	223	0.32

First calculation based on ♂ specimen in which both inulin space and muscle composition was measured; second calculation using mean inulin space (22.8 per cent of muscle water) and mean data for other two ♂ specimens.

Average water content: cells 727 g/kg, serum 951 g/liter.

(4) *Composition of serum before and after dialysis against sea water.* Since *Limulus* blood contains the protein haemocyanin and the gills are apparently permeable to the inorganic ions of sea water, it is possible that a Donnan equilibrium exists with respect to at least certain ions, those cations in higher concentration in the plasma than in sea water, and those anions in lesser concentration in the plasma.

A sample of serum in Visking viscose cellulose tubing was dialyzed against sea water of almost exactly the same concentration as that from which the animal was taken. After dialysis for 24 hr, the serum was analyzed for comparison with a sample of the original serum. The final pH of the sea water was 8.0.

For the cations and anions, excluding calcium, of the dialyzed serum, the mean Donnan ratio is 1.018 (Table VII). The much higher value of calcium in the serum, 115.5%, of that in the sea water, indicates the presence of some calcium bound in an unionized complex with protein. This is a usual feature in blood containing significant quantities of protein. A small proportion of magnesum may be bound, about 4% of the total, if the mean Donnan ratio is applied to the figure of 108.0%, the concentration of magnesium in the serum after dialysis. A slight anomaly is apparent, in that the chloride of the dialyzed serum expressed in relation to water content was never less than the chloride of the sea water expressed similarly, whereas it should have been nearly 2% less according to the Donnan relationship. This occurred despite careful replicate analyses.

Taking comparison of original serum with dialyzed serum as a measure of ionic regulation, it is clear that in the blood of the specimen analyzed sodium and potassium are regulated at values higher than those of physico-chemical equilibrium (sodium only slightly), that calcium, magnesium and sulphate are regulated at levels lower than those of equilibrium, leaving chloride as the only ion in diffusion equilibrium.

TABLE VII

Composition of serum before and after dialysis against sea water

	mg-ions/kg water						g/liter
	Na	K	Ca	Mg	Cl	SO_4	Water
Original serum	454	11.3	10.2	44.3	519	16.0	951
Serum after dialysis	450	9.5	11.2	54.3	517	25.4	950
Sea water	441	9.4	9.7	50.3	515	26.5	990
Serum as per cent dialyzed serum	100.9	118.9	91.1	81.6	100.4	63.0	—
Dialyzed serum as per cent sea water	102.0	101.1	115.5	108.0	100.4	95.8	—
Donnan ratio*	1.02	1.01	—	1.04	1.00	1.02	—

* $\frac{Na_s}{Na_{sw}} = \frac{\sqrt{Mg_s}}{\sqrt{Mg_{sw}}} = \frac{Cl_{sw}}{Cl_s}$ *etc.* Protein = 40.0 g/liter. Mean ratio = 1.018.

By considering also the other two detailed analyses of *Limulus* serum in Table IV, a general conclusion would be that *Limulus* regulates the ionic composition of its blood by reducing its calcium, magnesium and sulphate on the average some 12%, 12% and 43%, respectively, below the values obtaining in a physico-chemical equilibrium with sea water, while maintaining sodium and chloride within about 1–2% of equilibrium. Potassium shows the most variable regulation at about 24% above dialysis equilibrium (range 11–39%). Possible participation of the coxal glands and the gills in this ionic regulation has not been investigated.

(5) *Effect of salinity on composition of serum and muscle.* When *Limulus* is allowed to come into equilibrium with sea water of greater strength than normal, the blood becomes isosmotic (Table I). In Table VIII it is seen that in 150% sea water the pattern of ions in the serum is very similar to that in normal sea water, only potassium and calcium showing more than slight changes, in both cases a relative fall in level, more pronounced in the case of the latter. However, when in 200% sea water, the differences in ions between internal and external media practically disappear, essentially remaining only in sulphate concentration which in the serum is 85% of that in sea water, compared to 53% and 55% in 100% and 150% sea water.

TABLE VIII

Changes in composition of serum in relation to salinity of external medium

Sea water (per cent)	Medium	mg-ions/kg water						
		Na	K	Ca	Mg	Cl	SO_4	Total ions
200	Serum	921	19.7	23.3	103.5	1079	45.9	2192
	Sea water	924	19.7	22.9	102.3	1087	54.3	2210
	Serum as per cent sea water	100	100	102	101	99	85	99
150	Serum	627	15.0	13.2	62.7	721	20.5	1459
	Sea water	624	13.8	15.4	71.5	736	37.4	1498
	Serum as per cent sea water	100	109	86	88	98	55	97
100	Serum	445	11.8	9.9	46.0	514	14.1	1041
	Sea water	440	9.4	9.7	50.2	514	26.5	1050
	Serum as per cent sea water	101	126	102	92	100	53	99
50	Serum	297	5.69	6.86	28.1	326	6.7	670
	Sea water	225	4.78	4.93	25.7	263	13.5	537
	Serum as per cent sea water	132	119	139	109	124	50	125
20	Serum	224	7.88	5.66	13.7	222	2.5	476
	Sea water	87.4	1.86	1.92	10.0	102	5.3	209
	Serum as per cent sea water	256	424	295	137	218	47	228
10	Serum	156	3.80	3.28	6.5	153	2.0	325
	Sea water	45.8	0.98	1.00	5.2	53.5	2.8	109
	Serum as per cent sea water	341	390	328	125	286	71	298

Figures are means of analyses of two animals in 200 per cent sea water, of three in 100 per cent, and of single specimens in the other concentrations.

In the three diluted sea waters, the serum remains hyperosmotic, the percentage difference in total ions between it and the sea water increasing with dilution from 25% to 198%. Individual ions in the serum do not vary in accordance with the total concentration, each being apparently controlled separately. Sodium and chloride form 93–95% of the total ions in the serum (91–92% in 100% sea water and greater strengths). With dilution sodium rises slightly as a proportion of the total, chloride remaining nearly proportional. In the lower salinities serum potassium and calcium increase relatively more than sodium (although these ions fall in absolute concentrations), while magnesium and sulphate fall considerably in both absolute and relative terms.

Muscle concentrations of inorganic ions rise or fall with the concentration of the blood and the surrounding medium (Table IX). It is easy to visualize, say, an increase in salinity causing increases in blood ions which are then reflected by a final higher concentration of muscle ions; and similarly for decreases. But the concentrations of phosphate are more difficult to relate to the osmotic concentration of the blood. Being for the most part not easily diffusible compounds (except inorganic P), phosphates would be expected to be more stable in amount at different salinities, and certainly should not change on account of the negligible effect of minute quantities of phosphate in sea water. From the data for phosphorus in Table IX, it would appear that there is little fall in concentration in 20% and 10% sea waters; as salts are lost in the low salinities the phosphate rises as a percentage of total ions from 15% in full-strength sea water to 31% of the total in 5%. Although the absolute amount of phosphate, 132 mg-atoms, in one of the 200% specimens shows an 80% rise from the average of 73.2 in normal animals, it falls as percentage of the total ions to 10%.

It is this increase of phosphate to 132 and 137 mg-atoms that is puzzling, since it is far more than would be expected from the decrease in water content of the muscle, unless the specimens had, in normal sea water, higher muscle values than the highest (89.6 mg-atoms) of the 3 specimens with 73.3 mg-atoms as mean. While this is not impossible, phosphate may perhaps have been transferred to the muscle cells from other tissues, such as the hepatopancreas.

TABLE IX

The composition of muscle in relation to serum as influenced by salinity of external medium

Sea water (per cent)	Tissue	mg-ions/kg water							g/kg or g/l
		Na	K	Ca	Mg	Cl	P*	Total ions	Water
200	Muscle (1)	362	181	15.4	53.9	568	132	1312	663
	(2)	370	183	17.3	50.3	—	137	(757)	659
	Serum (1)	921	18.7	21.4	102.7	1064	0.31	2128	929
	(2)	921	20.7	25.2	104.3	1094	0.47	2166	945
	Ratio: $\frac{\text{muscle}}{\text{serum}}$ (1)	0.393	9.68	0.720	0.525	0.534	426	0.617	—
	(2)	0.402	8.84	0.687	0.482	—	291	—	—
	Mean	0.398	9.26	0.704	0.504	0.534	359	0.617	—
100	Muscle Mean†	126	99.5	3.76	20.5	159	73.3	482	763
	Serum Mean†	445	11.8	9.9	46.0	514	0.43	1027	951
	Ratio: $\frac{\text{muscle}}{\text{serum}}$	0.283	8.43	0.380	0.446	0.309	170	0.469	—
20	Muscle	46.1	89.5	1.88	12.6	47.4	76.0	273	807
	Serum	224	7.88	5.66	13.7	222	0.43	474	908
	Ratio: $\frac{\text{muscle}}{\text{serum}}$	0.206	11.35	0.332	0.92	0.214	177	0.576	—
10	Muscle	32.8	59.3	1.66	9.5	36.5	63.1	203	815
	Serum	156	3.80	3.28	6.5	153	0.35	323	974
	Ratio: $\frac{\text{muscle}}{\text{serum}}$	0.210	15.6	0.51	1.46	0.239	180	0.628	—

* Mg-atoms trichloracetic acid—soluble P.
† From Table IV.

Muscle/serum ratios in the altered sea waters show the same general features as the ratio in normal sea water, high values for potassium and phosphate, low values for the remaining ions. As salinity and serum concentrations decline, so do the muscle/serum ratios of sodium and chloride, but from a base-line of 100% sea water the ratios of potassium, magnesium and phosphate increase with dilution, that of phosphate only slightly. The end result is a slight increase from 0.47 to 0.63 in the ratio of muscle/serum total ions.

It is obvious that the ratios between the ions of muscle and serum are kept more constant over the range of salinity tested than the ions themselves. The highest ratios are never more than two or three times those of the lowest, but individual values for ions vary widely. For example, comparing muscle from *Limulus* in

10% and 200% sea waters, the highest values of sodium, calcium and chloride are, respectively, eleven, ten and sixteen times those of the lowest. This greater constancy in the ratios is due to the fact that the ions of the serum are varying in the same direction, influenced by the external salinity, and in fact are broadly responsible for the changes in muscle ions.

Nitrogenous constituents of muscle

In Table X are shown analyses of nitrogenous compounds and ammonium ions in the muscles of some of the same animals for which data on inorganic ions and total phosphate have already been given (Tables IV and IX). Total non-protein nitrogen of muscle, expressed as mg-atoms, does not differ much in the animals from 200% and 100% sea water, but is smaller in amount in the two animals in dilute sea water. The figures for the 3 specimens in normal sea waters, 538–757 mg-atoms, agree well with those of Bricteux-Grégoire *et al.* (1966), 462–776, although the latter are for hydrolyzed dialysates of homogenized muscle, whereas the figures in Table X are for tungstic acid extracts of muscle. In dilute sea waters the values are 406 and 235 (Table X) which may be compared with one of 271 mg-atoms given by the Belgian authors for an animal adapted to sea water of Δ 0.79° C (about 44% of concentration of Woods Hole sea water).

TABLE X

Nitrogenous constituents of muscle

Sea water (per cent)	mM/kg water						
	Free amino acids			Trimethylamine oxide	Urea	Ammonium	Total non-protein N†
	Total α-amino N	Arginine*	Proline				
200	316.7	—	55.7	—	—	—	743
100 (1)	82.3	27.2	2.7	3.9	0.8	6.7	538
(2)	128.1	61.2	25.1	4.9	0.4	2.9	756
(3)	103.4	41.5	6.9	5.2	—	5.6	757
Mean	104.6	43.3	11.6	4.7	0.6	5.1	684
20	88.5	49.4	5.4	4.4	0.5	4.1	406
10	67.6	25.5	0.6	1.7	1.1	3.1	235

* Probably mostly as arginine phosphate in the living animal.
† Mg-atoms.

A surprising feature is the relatively low concentration of free amino acids, an average of 105 m*M* in *Limulus*, compared with much higher values in decapod crustaceans (such as 512 m*M* in *Nephrops* (Robertson, 1961)). Included with the amino acids is taurine whose α-amino N reacts with Folin's amino acid reagent. The ratio of amino acids to total nitrogen increases in the muscles of animals in 200% sea water and in 10%. After taurine in concentration come arginine and proline, the latter rather variable, the former more consistent with values between 26 and 61 m*M*. Compared with *Nephrops*, *Limulus* has a small amount of proline in its muscles, and about half the amount of arginine.

Urea is present only in trace quantities and trimethylamine oxide to the extent of 5 m*M*. Ammonium ions are also present, but in small quantity. Some may have arisen by release of ammonium from compounds such as amides after the muscle extracts had been made.

Allowing for the 4 N atoms of arginine, and adding to the amino acid- and taurine-N the other estimated N- compounds plus ammonium, the sum still comes far below the total non-protein N, leaving 64% of it undetermined in the animals in full-strength sea water, 39% and 36% undetermined in the 20% and 10% diluted waters. Even taking into account the 5 N atoms of the adenosine phosphates which were not estimated, there would still remain more than half of the total N atoms still unallocated to known compounds in the muscles of animals in normal sea water, and about a quarter in the case of the specimen in 20% sea water.

Bricteux-Grégoire *et al.* (1966) who studied some *Limulus* in full-strength sea water (Δ 2.09° C) and in diluted sea water (Δ 0.78° C) have given details of individual amino acids in blood serum (total 0.7–1.5 m*M*/liter) and muscle. They also point to the small contribution of amino acids and taurine to the total dialyzable N, leaving unknown nitrogenous compounds playing a significant role in maintaining the osmotic concentration of the cells.

Osmolality

From the data given in the two previous major sections, the osmolality of serum and muscle can now be calculated at 4 salinities and that of muscle cells at normal salinity. Neglecting for the present the osmotic coefficients of the salts and nitrogenous compounds, and the possibility of some ion binding to proteins, a summation of inorganic and organic ions and of nitrogenous constituents should give osmolality, as the concentrations are per kg solvent water (Table XI).

TABLE XI

Osmotic concentration of serum, whole muscle and muscle cells

Constituent	mg-ions or mM/kg water								
	200 per cent sea water		100 per cent sea water			20 per cent sea water		10 per cent sea water	
	Serum	Muscle	Serum	Muscle	Muscle cells	Serum	Muscle	Serum	Muscle
Sodium	921	366	445	126.2	28.8	224	46.1	156	32.8
Potassium	19.7	182	11.8	99.5	129	7.9	89.5	3.8	59.3
Calcium	23.3	16.4	9.9	3.76	1.85	5.7	1.88	3.3	1.66
Magnesium	103.5	52.1	46.0	20.5	13.0	13.7	12.6	6.5	9.5
Ammonium	—	—	—	5.1	6.6	—	4.1	—	3.1
Chloride	1079	568	514	159	43.2	222	47.4	153	36.5
Sulphate	45.9	—	14.1	3.9	0.9	2.5	—	2.0	—
Lactate	—	—	0.31	19.1	22.7	—	21.3	—	6.5
Phosphate	0.39	135	0.43	73.3	95.7	0.43	76.0	0.35	63.1
Total ions	2193	1320	1041.5	510.4	341.8	476.2	298.9	325.0	212.5
Amino acids	—	317	—	105	136	—	89	—	68
Trimethylamine oxide	—	—	—	4.7	6.1	—	4.4	—	2.1
Urea	—	—	—	0.6	0.8	—	0.5	—	1.1
Remaining non-protein N	—	426	—	439	568	—	160	—	85
Total	2193	2063	1042	1060	1053	476	553	325	369

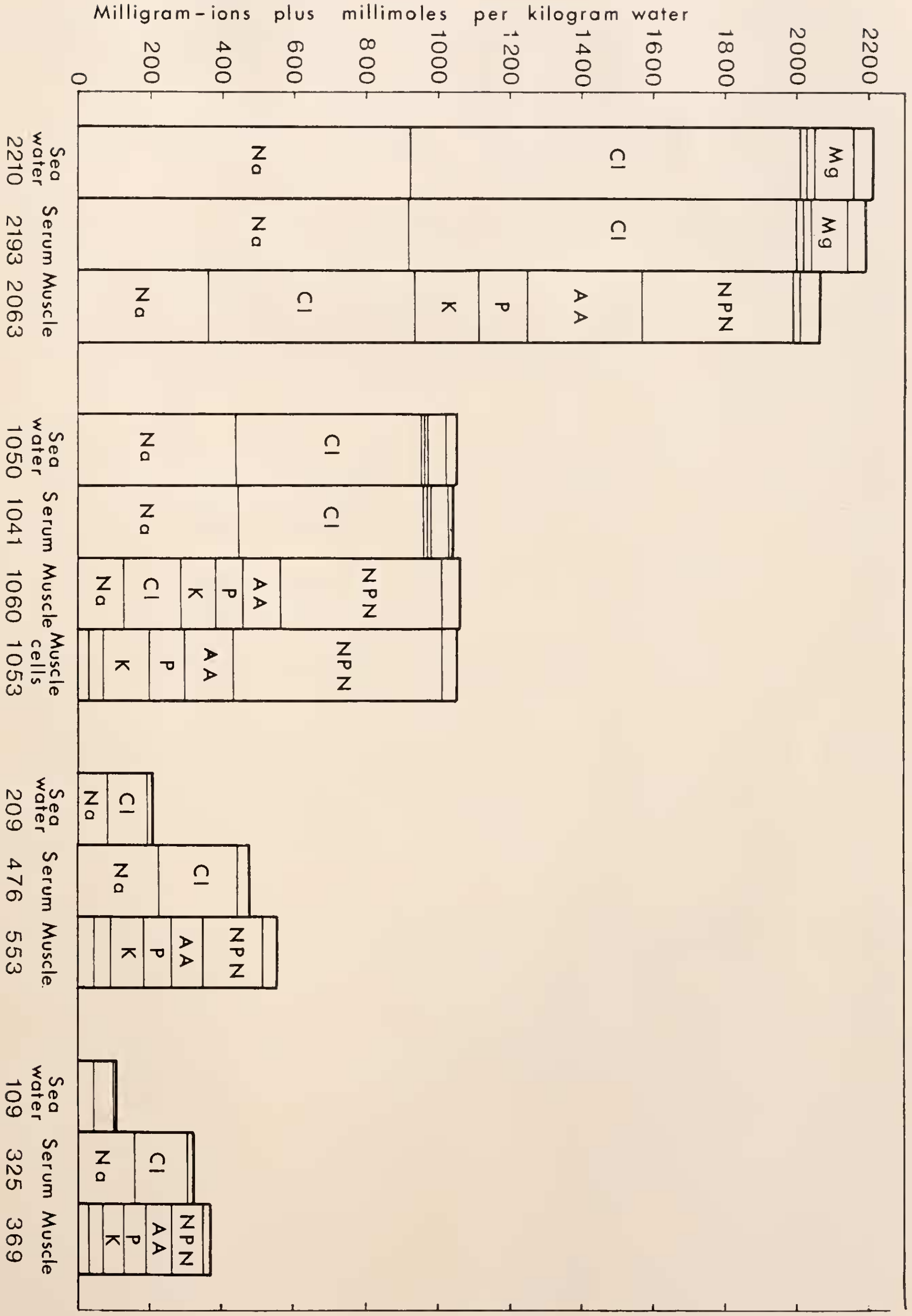

FIGURE 4. Composition of *Limulus* serum and muscle in relation to concentration of the external medium; AA—amino acids; NPN—remaining non-protein nitrogen.

The agreement is good (within 2%) between serum, muscle and muscle cell concentrations in the animals from 100% sea water, less good in the comparison of muscle and serum at the other salinities, in which the muscle concentrations exceed the serum by 14–16% in the specimens from dilute sea water, and are 6% below in the specimens from 200% sea water.

Figure 4 shows the changes in the principle constituents at the 4 concentrations studied.

Apart from the uncertainties of ion binding, several inaccuracies leading to overestimates exist in the muscle data: the phosphate is really in mg-atoms as the fraction of ATP (with its 3 P atoms) was not determined; the remaining non-protein N includes the 5 N atoms of ATP; any arginine phosphate is included both under amino acids and phosphate; the remaining non-protein N compounds may (like the adenosine polyphosphates) contain more than 1 atom N per molecule. So more complete knowledge would tend to reduce the total osmotic concentration of muscle as estimated.

A more accurate estimate of osmolality would be obtained if osmotic coefficients for sea water and for organic compounds are used (Robertson, 1961, 1965). Unfortunately, lack of published data on the osmotic coefficients of most of the amino acids makes this impossible even when the concentrations of each amino acid in the muscle are known, and even the identity of the other nitrogenous compounds in muscle has not been established. Perhaps it is best at present to assume an average coefficient of 1 for all the nitrogenous compounds.

For inorganic salts the osmotic coefficients are well below that for ideal solutions, and for sea water osmotic coefficients can be calculated from the osmotic equivalence data of Robinson (1954) and the g-ionic concentration of sea water (1.1368 at a chlorinity of 19.00‰) as given by Sverdrup, Johnson and Fleming (1942). For example, the osmotic coefficient for a sea water of Cl 19.00‰ is 1027.3/1136.8, the first figure being the concentration in milliosmoles and the second figure milligram-ions by analysis. This value is 0.904, and corresponding values for sea waters of Cl 22.00‰ and 10‰ are 0.908 and 0.897. For 200% sea water the osmotic coefficient may be calculated to be 0.92.

It is reasonable to assume that the predominantly inorganic ions of *Limulus* serum have proportionally the same osmolar concentration as the ions of sea water and that the appropriate osmotic coefficients can be applied. It is further assumed that the inorganic ions and phosphates of the muscle would behave approximately like serum ions. In the calculations of the milliosmolar concentrations of Table XII no allowance has been made for possible ion binding. This is negligible in the serum, but probably significant in the muscle; its extent in muscle remains a subject for future research. The final calculations show that in all cases except the 200% sea water specimens (for which only one N-analysis is available) the muscle has an apparently higher osmotic concentration than the serum, about 7–8% higher in both muscle cells and whole muscle, some 18–22% higher in the specimens from 10% and 20% sea water. Possible reasons for this have already been discussed. There is no reason to doubt that the blood and muscle are isosmotic. Direct measurements of osmolality of the sera by the Krogh-Baldes thermoelectric method show good agreement with the calculated totals. The slightly lower totals would have been increased by a few milliosmoles if estimations had been made of amino acids (*ca.* 0.7–1.6 m*M*, Bricteux-Grégoire *et al.*, 1966) glucose (*ca.* 0.2 m*M*, Tamar, 1965) and bicarbonate (7 mg-ions, Homer W. Smith in Cole, 1940).

Discussion

When comparisons are carefully made of the blood of *Limulus* which have been equilibrated with normal Woods Hole sea water, 31–32‰, the serum has been found to be isosmotic within 1% (freezing-point data of Garrey, 1905 and of Dailey, Fremont-Smith and Carroll, 1931, also the present data using a thermoelectric vapor pressure method). Cole (1940) states that 6 pooled samples of *Limulus* blood collected over 3 summers were "consistently hypertonic" and thinks that this is the normal condition. The quoted values for animals kept 3 months in Salsbury Cove (Maine) sea water are serum Δ 1.783° C, sea water 1.759° C, a difference of 1.4%. He also gives data for specimens from the New York Aquarium analyzed by Smith, serum Δ 1.880° C, sea water 1.850° C, a difference of 1.6%. It is difficult to judge the weight to be given to these values since, if they are means, no tests of significance are given or estimates of errors of measurement. Although no differences between the sexes were found in the osmotic concentration of the blood from animals in 100% sea water (Table I), there is a significant difference at the level of $P = 0.05$ between the means of the 10 values for sea water, 951 ± 0.298 and those for serum, 955 ± 1.303. Thus the blood is slightly hyperosmotic, by 0.4%, and it may have been approximately so in the specimens studied by Cole and Smith. A conclusion that the blood of *Limulus* is isosmotic with sea water of salinity 31–34‰ within 1.5% would practically cover the data.

Table XII

Osmolality of serum, whole muscle and muscle cells

	milliosmoles/kg water								
	200 per cent sea water		100 per cent sea water			20 per cent sea water		10 per cent sea water	
	Serum	Muscle	Serum	Muscle	Muscle cells	Serum	Muscle	Serum	Muscle
Inorganic ions and phosphate *etc.*	2018	1214	940	461	309	429	269	293	191
Amino acids	—	317	—	105	136	—	89	—	68
Trimethylamine oxide and urea	—	—	—	5	7	—	5	—	3
Remaining non-protein N	—	426	—	439	568	—	160	—	85
Total	2018	1957	940	1010	1020	429	523	293	347
Direct measurement	2017	—	953	—	—	435	—	299	—

McManus (1969) using a comparative melting-point method found that horseshoe crabs from Delaware Bay were approximately isosmotic when kept in tanks of running bay water of about 25‰ salinity. At this salinity the osmotic equivalence should be 0.40 *M*-NaCl and from one of his figures the bay water seems to be only 0.34 *M*. He claims considerable fluctuations among his 8 large male specimens in this water, both among individuals and at different times of successive sampling (8, 16 and 24 hr). Two points on the figure suggest that the blood may become hypo-osmotic (0.31–0.32 *M*) after 8, 16 and 24 hr in the water. This seems very improbable, and shows, perhaps, a limitation in accuracy of the melting-point technique. Dailey *et al.* (1931) stress the importance of comparing the blood with sea water each time equilibrium studies are made, as they found that *Limulus* blood reflected the dilution of sea water with rain water at Woods Hole, remaining isosmotic.

McManus (1969) found that large male crabs could withstand direct transfer to diluted water of about 13‰ salinity without any ill effects, but transfer to 6–7‰ and to deionized water caused swelling of the limb joints and gills, with bursting of the gill membranes in 4 out of 8 of the former and in the 4 latter. Osmotic concentrations measured up to 24 hr showed a rapid fall in 13‰ and 6–7‰ salinity but remained higher than the medium. Four specimens (118–479 g) transferred gradually from 25‰ to 3–4‰ salinity, with stays of 4 days in 13‰, and 2 days in 6–7‰ changed little in weight over this period, and became noticeably sluggish in the final dilution, although maintaining normal weight within 2% for 74 hr.

A salinity tolerance study on groups of 10 small *Limulus* (prosomal width 7.5 cm or less) over a period of 127 hr showed no deaths in diluted sea water of 12.5‰, but a rising mortality of 5%, 15%, 30%, and 40% in waters of 10.0‰, 7.5‰, 5.0‰ and 2.5‰, respectively, while the 20 kept in deionized water all died in 12–72 hr.

Apart from McManus's study the only published data on osmotic regulation in *Limulus* are isolated values such as those of Cole (1940) on a specimen from Delaware Bay, Δ 1.378° C, sea water 1.336° C; and on one kept in New York Harbor sea water, serum Δ 1.01° C, water 0.651° C (analyzed by Homer W. Smith). Bricteux-Grégoire *et al.* (1966) reported the mean value of Δ 0.85° C for medium sized specimens gradually acclimatized to diluted sea water of Δ 0.79°. At this sea water strength they survived at least 15 days. Garrey (1905) gave values of Δ 1.12° C in a specimen after 52 hr in sea water of Δ 1.02° C, and also found that the blood of a specimen kept for 16 hr in sea water concentrated by evaporation to give Δ 3.80° C was isosmotic at Δ 3.79° C.

The foregoing data are widely expanded and reinforced by the osmotic concentration figures given in Table I, from animals in 5–200% sea water, 47–2084 milliosmoles (about Δ 0.09°–Δ 3.87° C). *Limulus* is extremely tolerant of changes in salinity. Over a sea water range of 100–200% the blood concentrations are 954–2058 milliosmoles, the blood being isosmotic within 1.5%, while over the 5–66% range the blood is hyperosmotic, ranging between 176 and 718 milliosmoles in water of 47–630 milliosmoles.

If the osmotic concentration curve (Figure 1) is compared with that for the euryhaline decapod crustacean *Carcinus maenas*, common in European coastal waters and the Woods Hole region, the osmotic tolerance as determined experimentally is seen to be much the same, Δ 0.17° C–Δ 2.88° C (91–1550 milliosmoles) (Robertson, 1960b), but at the lowest salinity studied by Duval (1925), Δ 0.17° C, only 2 of 6 crabs survived for 20 hr, and those in water of Δ 0.38° C died in 24–48 hr. These were all direct transfer experiments with no intermediate acclimatization. In water of Δ 0.60° C (323 milliosmoles) and above the crabs seemed to survive indefinitely.

More recent studies of *Carcinus* from the North Sea (32‰) and the Baltic (13–18‰) have shown that in water of about Δ 0.33° C (178 milliosmoles) animals from the Baltic had the greater freezing-point depressions after 3 days, equivalent to about 538–754 compared with 452–592 milliosmoles. Cross adaptation to different habitat salinities for 2–3 weeks reduced but did not abolish these differences (Theede, 1969).

The main difference in osmoregulatory ability between *Carcinus* and *Limulus* is that the former maintains its blood at relatively higher osmotic concentration in low salinities. Theede's data are for a temperature of 16.5° C, Duval's (unspecified) probably at temperatures near those of *Limulus* in this paper, 21–23° C.

The San Francisco Bay population of *Rhithropanopeus harrisi*, a xanthid crab, is well adapted to brackish water situations. The melting-point value of the blood at a salinity of about 21 m*M*-NaCl, 39 milliosmoles, is near 232 m*M*, about 424 milliosmoles (Smith, 1967), a concentration considerably higher than the 220–335 maintained by small *Limulus* in a medium of 47–48 milliosmoles.

As mentioned in the introduction, *Limulus* has sometimes been found in waters of salinity 6–8‰ (18–24% of Woods Hole concentration). Whether these are young or adult animals, or whether the stay in such diluted water is a seasonal phenomenon in the particular areas, it seems from the data in Table I that even lower salinities could be tolerated for some time, especially if the animals faced a gradual acclimatization.

Present and former analyses of blood-serum of *Limulus* are compared in Table XIII. Considering the varied methods by which the results were obtained and the possibility of systematic and other errors, the results are not too different. Macallum's (1910) analysis mostly in triplicate, using classical gravimetric techniques, is perhaps closest to those in this paper. Cole (1940) admits a systematic error in his magnesium method leading to low results (−30% in the analysis of sea water) and he attributes values of 0.0629 and 0.0609 given by Smith for the SO_4/Cl ratio in Salsbury Cove and New York sea water (+22% and 18% on the standard accepted value) as perhaps due to normal variation in near-shore sea water. This is improbable. Cole's Na/Cl ratio for sea water, 0.931, is too high by 9% and so possibly are his ratios for *Limulus* serum. For the methods used by the author a series of estimations on synthetic sea waters gave standard deviations from the theoretical for Na, K, Ca, Mg, Cl and SO_4 of 0.28%, 1.04%, 0.81%, 1.36%, 0.06% and 0.60%, respectively.

In diluted sea waters the general finding is a decrease in the Mg/Cl and SO_4/Cl ratios and an increase in that of Na/Cl. The K/Cl and Ca/Cl ratios of the serum remain larger than the respective sea water ratios.

If ionic regulation is defined as the maintenance of concentrations of ions differing from those of a passive equilibrium with the external medium, the pattern in *Limulus* (Table VII) is closest to that of the spider crab *Maia squinado* of the 14 decapod crustaceans examined in the same way (Robertson 1949, 1953). Only in its low calcium level, lower than that of dialysis equilibrium, does it differ from *Maia* and the other isosmotic decapods. This low level of calcium seems to be associated with lack of a calcified skeleton in *Limulus*.

In muscle of *Limulus* from normal sea water, inorganic ions and organic phosphates and lactate account for about 48% of the osmotic concentration, the remaining proportion being made up by non-protein nitrogenous compounds. This figure is practically identical with that in *Nephrops* (Robertson, 1961), but after subtraction of the extracellular fluid the proportion of ions in the muscle cells falls to 32% in *Limulus* compared to 40% in *Nephrops*. In animals from reduced salinities, the proportion increases to 54% and 58% in 20% and 10% sea waters, and also to 64% in animals from 200% sea water.

TABLE XIII

Composition of Limulus *blood-serum, according to various authors, milligram-ion* (*m*M) *ratios in relation to chloride*

Source of material and author	Cl (mg-ions/l)	Na/Cl	K/Cl	Ca/Cl	Mg/Cl	SO_4/Cl
Woods Hole Macallum, 1910	487	0.824	0.0272	0.0192	0.0873	0.0315
Woods Hole Dailey *et al.*, 1931	500	0.883	—	—	—	—
Maine Cole, 1940	478	0.956	0.0207	0.0195	0.0605	0.0442
New York Smith in Cole, 1940	463	0.909	0.0311	0.0346	0.0584	0.0445
Woods Hole	489	0.866	0.0230	0.0193	0.0895	0.0274
J. D. R.	310	0.958	0.0184	0.0221	0.0906	0.0216
Woods Hole	202	1.009	0.0355	0.0255	0.0617	0.0113
J. D. R.	149	1.020	0.0248	0.0214	0.0425	0.0131
Delaware Bay Cole, 1940	308	0.984	0.0282	0.0205	0.0799	0.0183
New York Harbor Smith in Cole, 1940	265	0.981	0.0249	0.0208	0.0194	0.0169
Sea water Sverdrup *et al.*, 1942	549	0.856	0.0181	0.0187	0.0976	0.0515

Decrease in salinity leads to a reduction in the amounts of non-protein nitrogenous constituents in *Limulus* muscle. This is a feature found also in euryhaline crustaceans (*e.g.*, *Carcinus*, Shaw 1958; *Eriocheir*, Bricteux-Grégoire *et al.* 1962) in which free amino acids may account for a third or a half of the osmotically active constituents in muscle. This proportion in *Limulus* is much less, as pointed out already by Bricteux-Grégoire *et al.* (1966), 6–9% and 10% (including taurine) in the muscles of their specimens in full strength and 40% sea water, and 12–23% in the present series over 200–10% sea water. This leaves a large fraction of the non-protein nitrogen still undetermined and unknown, as it must be attributed to compounds other than trimethylamine oxide and urea.

Finally, the wide tolerance of *Limulus* to increases and decreases of salinity must be of value to animals temporarily cut off in evaporating lagoons or shallows in the southern part of its range or in areas affected by freshwater outflows. Information is required on the salinity tolerance of younger stages of *Limulus* than those studied here or by McManus (1969), and on whether the appearance of specimens in brackish water is related to such factors as season, temperature and state of maturity which are likely to influence powers of osmotic regulation.

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SUMMARY

1. Osmotic and ionic regulation of *Limulus* has been studied over a wide range of salinities. The blood is isosmotic in sea water of 100–200% (950–2084 milliosmoles) within 1.5%. It is hyperosmotic in dilute sea water of 10–66%. After a period of acclimatization in waters of 50–10%, some small horseshoe crabs (prosomal width about 7 cm) remained healthy in 5% sea water (48 milliosmoles, about 1.7‰ salinity) for 2–5 days, the blood in 4 specimens being kept at a level of 229–335 milliosmoles.

2. Analyses of blood-serum as originally obtained and after dialysis against sea water give the following measure of ionic regulation: Na 101%, K 119%, Ca 91%, Mg 82%, Cl 100% and SO_4 63%.

3. Weight changes in waters of different salinity have been studied and the final water content of the muscles. The latter ranges from 67% in specimens from 200% sea water to 83% in specimens from 10%.

4. From experiments with injected inulin solutions, the average extracellular volume in crabs of 100–634 g weight is 52% of the body weight.

5. Estimates of extracellular water in the muscles (22.8% of total muscle water) have been used for calculations of intracellular concentrations of inorganic ions, phosphates and various non-protein nitrogenous compounds. Two-thirds of the osmotic concentration of the muscle cell is made up by the latter, of which free amino acids constitute only a small fraction.

6. In whole muscle of specimens kept in 20% and 10% sea water the absolute amounts of ions and molecules fall in conformity with blood concentration, the ions increasing as a proportion of the serum concentration from 47% in 100% sea water to 58% and 63%, respectively. In 200% sea water ions in muscle as a proportion of those in serum also rise, to 62%.

LITERATURE CITED

ABEL, O., 1927. *Lebensbilder aus der Tierwelt der Vorzeit* [2nd ed.] Gustav Fischer, Jena, 714 pp.

AYERS, J. C., 1959. The hydrography of Barnstable Harbor, Massachusetts. *Limnol. Oceanogr.,* **4**: 448–462.

Bricteux-Grégoire, S., Gh. Duchâteau-Bosson, Ch. Jeuniaux and M. Florkin, 1962. Constituants osmotiquement actifs des muscles du crabe chinois *Eriocheir sinensis,* adapté à l'eau douce ou à l'eau de mer. *Arch. Int. Physiol. Biochem.,* **70**: 273–286.

Bricteux-Grégoire, S., Gh. Duchâteau-Bosson, Ch. Jeuniaux and M. Florkin, 1966. Les constituants osmotiquement actifs des muscles et leur contribution à la régulation isosmotique intracellulaire chez *Limulus polyphemus. Comp. Biochem. Physiol.,* **19**: 729–736.

Cole, W. H., 1940. The composition of fluids and sera of some marine animals and of the sea water in which they live. *J. Gen. Physiol.,* **23**: 575–584.

Conway, E. J., 1962. *Microdiffusion Analysis and Volumetric Error.* [5th ed.] Crosby Lockwood, London, 467 pp.

Dailey, M. E., F. Fremont-Smith and M. P. Carroll, 1931. The relative composition of sea water and of the blood of *Limulus polyphemus. J. Biol. Chem.,* **93**: 17–24.

Dick, D. A. T., 1959. Osmotic properties of living cells. *Int. Rev. Cytol.,* **8**: 387–448.

Duval, M., 1925. Recherches physico-chimiques sur le milieu intérieur des animaux aquatiques. Modifications sous l'influence du milieu extérieur. *Ann. Inst. Océanogr.,* **2**: 233–407.

Ennor, A. H., 1957. Determination and preparation of N-phosphates of biological origin. II. Phosphoarginine, pp. 857–861. *In:* S. P. Colowick and N. K. Kaplan, Eds., *Methods in Enzymology, Vol. 3.* Academic Press, New York.

Garrey, W. E., 1905. The osmotic pressure of sea water and of the blood of marine animals. *Biol. Bull.,* **8**: 257–270.

Heagy, F. C., 1948. The use of polyvinyl alcohol in the colorimetric determination of magnesium in plasma or serum by means of titan yellow. *Can. J. Res. Series E,* **26**: 295–298.

Howarth, M. K., 1964. The Jurassic period, pp. 203–206. *In:* W. B. Harland, A. G. Smith and B. Wilcock, Ed., *The Phanerozoic Time-Scale.* Geological Society of London, London.

Janus, J. W., 1956. Determination of arginine. *Nature,* **177**: 529.

Krogh, A., 1939. *Osmotic Regulation in Aquatic Animals.* University Press, Cambridge, 242 pp.

Lochhead, J. H., 1950. *Xiphosura polyphemus,* pp. 360–381. *In:* F. A. Brown, Ed., *Selected Invertebrate Types.* Wiley, New York.

Macallum, A. B., 1910. The inorganic composition of the blood in vertebrates and invertebrates, and its origin. *Proc. Roy. Soc. London Series B,* **82**: 602–624.

McManus, J. J., 1969. Osmotic relations in the horseshoe crab, *Limulus polyphemus. Amer. Midland Natur.,* **81**: 569-573.

Pearse, A. S., 1928. On the ability of certain marine invertebrates to live in diluted sea water. *Biol. Bull.,* **54**: 405–409.

Robertson, J. D., 1949. Ionic regulation in some invertebrates. *J. Exp. Biol.,* **26**: 182–200.

Robertson, J. D., 1953. Further studies on ionic regulation in marine invertebrates. *J. Exp. Biol.,* **30**: 277–296.

Robertson, J. D., 1960a. Studies on the chemical composition of muscle tissue. I. The muscles of the hagfish *Myxine glutinosa* L. and the Roman eel *Muraena glutinosa* L. *J. Exp. Biol.,* **37**: 879-888.

Robertson, J. D., 1960b. Ionic regulation in the crab *Carcinus maenas* (L.) in relation to the moulting cycle. *Comp. Biochem. Physiol.,* **1**: 183–212.

Robertson, J. D., 1961. Studies on the chemical composition of muscle tissue. II. The abdominal flexor muscles of the lobster *Nephrops norvegicus* (L.). *J. Exp. Biol.,* **38**: 707–728.

Robertson, J. D., 1965. Studies on the chemical composition of muscle tissue. III. The mantle muscle of cephalopod molluscs. *J. Exp. Biol.,* **42**: 153–175.

Robinson, R. A., 1954. The vapour pressure and osmotic equivalence of sea water. *J. Mar. Biol. Ass. U. K.,* **33**: 449–455.

Robinson, R. A., and R. H. Stokes, 1959. *Electrolyte Solutions.* Butterworths, London, 571 pp.

Roe, J. H., J. H. Epstein and N. P. Goldstein, 1949. A photometric method for the determination of inulin in plasma and urine. *J. Biol. Chem.,* **178**: 839-845.

SCATCHARD, G., AND S. S. PRENTISS, 1933. The freezing points of aqueous solutions. IV. Potassium, sodium and lithium chlorides and bromides. *J. Amer. Chem. Soc.*, **55**: 4355–4362.

SHAW, J., 1958. Osmoregulation in the muscle fibres of *Carcinus maenas*. *J. Exp. Biol.*, **35**: 920–929.

SHUSTER, CARL N., JR., 1950. Observations on the natural history of the American horseshoe crab, *Limulus polyphemus*. *Third report on investigations of methods of improving the shellfish resources of Massachusetts.* Contribution No. 564, Woods Hole Oceanographic Institution, Dec. 31, 1950.

SHUSTER, CARL N., JR., 1957. Xiphosura (with especial reference to *Limulus polyphemus*). *Geol. Soc. Amer., Mem.* **67**(1), 1171–1173.

SHUSTER, CARL N., JR., 1960. Xiphosura. *In: Encyclopedia of Science and Technology* [McGraw-Hill Book Co., Inc.] **14**: 563–567.

SMITH, R. I., 1967. Osmotic regulation and adaptive reduction of water-permeability in a brackish-water crab, *Rhithropanopeus harrisi* (Brachyura, Xanthidae). *Biol. Bull.*, **133**: 643–658.

STØRMER, L., 1952. Phylogeny and taxonomy of fossil horseshoe crabs. *J. Paleontol.*, **26**: 630–640.

SVERDRUP, H. U., M. W. JOHNSON AND R. A. FLEMING, 1942. *The Oceans.* Prentice Hall, New York, 1087 pp.

TAMAR, H., 1965. Absence of hyperglycemic response at or after ecdysis in *Xiphosura polyphemus*. *Biol. Bull.*, **129**: 426–427.

THEEDE, H., 1969. Einige neue Aspekte bei der Osmoregulation von *Carcinus maenas*. [Some new aspects in the osmoregulation of *Carcinus maenas*]. *Marine Biology*, **2**: 114–120.